Original Article

Changes of Serum Level of Homocysteine and Oxidative Stress Markers by Metformin and Inositol in Infertile Women with Polycystic Ovary Syndrome: A Double Blind Randomized Clinical Trial Study

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Abstract

Background: Hyperhomocysteinemia plays an important role in the anovulation in infertile women suffering from polycystic ovary syndrome (PCOS). However, long-term metformin therapy elevated homocysteine (Hcy) concentration in these individuals. Inositol increases serum insulin levels and improves ovulation. The aim of this study was to compare the effect of metformin and inofolic on the level of serum Hcy and oxidative markers in the infertile patients with PCOS.

Materials and Methods: Eighty PCOS infertile women undergoing *in vitro* fertilization in the Umm-al-Banin clinical center, Dezful, Iran from December 2018 to September 2019 were invited to participate in this double blind randomized clinical trial. They were divided into two groups; group A who received metformin (1000 mg twice/day) and folic acid (400 µg /day) and group B who used inofolic (inositol+ olic acid 200 µg twice/day) for 3 months.

Results: The mean Hcy levels increased significantly by metformin (P=0.02), but not by inofolic. There was a decrease in the total antioxidant capacity (TAC) after metformin administration (P=0.01). In both groups, a significant increase in folic acid levels was observed after treatment (P=0.04). Also, no significant change in vitamin B12 and malondialdehyde levels was observed in both groups (P=0.08).

Conclusion: These findings indicate an increase in the serum Hcy levels as well as a remarkable decrease in TAC following metformin treatment. Given the rise in blood Hcy in PCOS patients, inofolic and other medications containing inositol can be prescribed instead of metformin (registration number: IRCT20190508043516N1).

Keywords: Homocysteine, Inofolic, Metformin, Oxidative Stress, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder that is responsible for a high proportion of infertility causes observed in 5-10% of women of reproductive age (1). PCOS cases are mostly characterized by the symptoms such as oligomenorrhea, hirsutism, anovulation (2), hyperandrogenism, hyperinsulinemia (following decreased tissue sensitivity to insulin) and high level of estrogen, which can impair the metabolic parameters and lead to the changes in lipid profile and oxidative stress biomarkers (3). Indeed, hyperinsulinemia can itself play a pathogenic role in this syndrome. In the long term it causes metabolic disorders such as glucose intolerance, type II diabetes and cardiovascular disease (CVD) (4). Therefore, previous studies have reported an elevated insulin rate in the serum of PCOS patients and subsequently a potent correlation was observed between insulin resistance and increased serum homocysteine (Hcy) levels in these women (5, 6). Hcy is an intermediary product of the methionine metabolism pathway and is catalyzed by the multiple enzymes along with the vitamin B12 and folic acid as a cofactor (7). Concentrations of Hcy in follicular fluid may be a useful marker in PCOS such as for fertilization rate, and oocyte and embryo quality (8).

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Metformin as an insulin sensitizer has been prescribed for PCOS patients. One study reported that metformin significantly increased Hcy level from 8.93 ± 0.49 to 11.26 ± 0.86 mmol/l in the serum of PCOS patients (9). It is possible that in diabetic patients, metformin administration can increase Hcy level by a reduction in the necessary cofactors such as plasma vitamin B12 and folic acid. While folic acid administration corrects Hcy elevation and vitamin B12 deficiency induced by metformin (5).

Folic acid (vitamin B9) controls blood Hcy levels (7). Its supplementation showed to prevent infertility and elevate ovulatory rates (10). Insulin sensitizers such as myoinositol have been advised to be prescribed for PCOS patients (11). This agent eventually stimulates the ovarian response to the endogenous gonadotropins by decreasing insulin secretion and androgens levels, therefore, enhance the ovulatory rates and spontaneous fertility (12). Studies support the idea of the insulin pathway impairment due to a defect in secondary inositol phosphoglycan (IPGs) (13). Also, in PCOS women deficiency or alteration in tissue inositol metabolism and IPGs may be due to insulin resistance (14). The supplementation with myoinositol and folic acid causes significant improvement in the serum level of insulin sensitivity and a reduction of Hcy after 3 months of treatment in PCOS participants (15). Also, a research concluded the use of myoinositol is capable of restoring ovarian function, metabolic and hormonal parameters, and subsequently fertility, in women with PCOS (16). Myoinositol therapy in women with PCOS causes better fertilization rates and a better embryo quality (17).

On the other hands, oxidative stress is an imbalance between reactive oxygen species production and the antioxidant defense system that ultimately causes oxidative damage to tissues. Total antioxidant capacity (TAC) is a combination of specific antioxidants such as vitamins E, C, and beta-carotene in the serum (18). Malondialdehyde (MDA) is also an indicator of lipid peroxidation and raise in the oxidative stress (19). Studies have designated an increase in oxidative stress in PCO patients (20). Some studies have reported that myoinositol is effective in reducing oxidative abnormalities in PCOS patients (21). Thus, metformin increases Hcy in women with PCOS (22). Therefore, we evaluated these two factors to investigate the effect of metformin and inofolic on the oxidative stress. However, to our knowledge there is no comprehensive investigation on the metformin consumption and its accompaniment with folic acid on the oxidative stress and Hcy in PCOS infertile women. Accordingly, this study aimed to investigate the effect of metformin and inofolic on the serum Hcy, folic acid and vitamin B12 levels in the PCOS infertile patients.

Materials and Methods

Subjects

This double blind randomized clinical trial was conducted on 80 infertile women with PCOS, who were elected with Block randomization method and attended for *in vitro* fertilization (IVF) or intra cytoplasmic sperm injection (ICSI) to the Umm-al-Banin clinical center in Dezful, Iran from December 2018 to September 2019. The PCOS diagnosis was based on two out of three Rotterdam criteria counting: clinical signs or biochemical excessive androgen level, oligomennerhea and/or anovulation, and morphology of ovaries in sonography termed as 12 or more small follicles (1).

The Ethics Committee of Dezful University of Medical Sciences approved the study protocol (IR.DUMS.REC.1397.037), registration ID in IRCT (IRCT20190508043516N1) and written informed consent was obtained from all the participants. Our inclusion criteria included women aged 22 to 38 years with a diagnosis of PCOS and serum levels of follicle-stimulating hormone (FSH) <10 IU / L on the third day of the cycle, minimum duration of infertility 3 years, who had no history of diabetes mellitus, hyperprolactinemia, congenital adrenal hyperplasia, thyroid disorders, cushing's syndrome, hypertension, folate and vitamin B12 deficiency, liver and kidney disorders.

All of the candidates <22 or >38 years, other causes of infertility, consumption of antioxidant drugs, were also excluded from the study. Also patients with current or previous history of using metformin within the last 3 months, other drug containing estrogen, progesterone and antiandrogenic effect such as combined oral contraceptive pill (OCP), antihypertensive drug were excluded

Intervention

This clinical trial study was conducted as a double-blind to remove probable biases in the evaluation of drug effectiveness. The research clinical group is responsible for concealment and allocation of the patients. Also, the samples will not know which drug product will be received. Only an Epidemiologist in the study was responsible for processing of the randomization and blinding to have access to blinded information on drug products. The other executive team will also remain as blind.

Patients were divided into two groups, 40 patients in each group, group A received 1000 mg metformin twice a day with 400 µg folic acid (Health Aid Co., UK) per day and group B consumed inofolic (LO.LI.pharma Co., Italy) 2 sachet per day (average amount per daily dose 1 sachet: Myo inositol 2000 mg, folic acid 200 µg) for a period of 12 weeks. Serum Hcy, MDA, TAC, vitamin B12, and folic acid levels were examined before and after consuming metformin and inofolic.

Outcome measured

Laboratory and biochemical analysis

Age, weight, body mass index (BMI, kg/m^2), menstrual pattern and laboratory study of homocysteine, folic acid, vitamin B12, malondialdehyde and TAC were measured in all of the participants for baseline parameters.

Women with cycles between 21-35 days considered regular cycle, 36 to 180 irregular cycles, cycles of less than 21 days of polymenorrhea, 36 to 180 days of oligomenorrhea, and cycles with intervals of 180 days or more were considered amenorrhea (2).

Blood samples were collected from each patient before and after treatment (12 weeks after beginning). Serums were separated immediately after sampling to avoid Hcy levels being increased and were stored at -70°C until assayed. Then Hcy levels were measured by UV Enzymatic Assay using the Hcy EIA Kit (DRG International Corporation, USA) with accuracy of 1 µmol /L.

Also, folic acid and vitamin B12 levels were measured by Cobas immunoassay (Roche Co., Germany). Oxidative stress factors (malondialdehyde and total plasma antioxidant) were respectively measured by Nalondi™ Lipid Peroxidation (MDA) assay. MDA assay has relied on a reaction with thiobarbituric acid (the TBARS assay) to generate a product that can be measured colorimetrically at 532 nm. Also, the total antioxidant defense was measured by Naxifer™ TAC assay kit with the ferric reducing ability of plasma (FRAP) assay.

Statistical analysis

The results were expressed as the mean ± SD. Difference between mean serum TAC, MDA, vitamin B12, folic acid, and Hcy levels before and after metformin or inofolic consumption was assessed by paired t test. All statistical tests were performed using the Statistical Package for the Social Sciences software (SPSS 16.0, SPSS Inc., Chicago, IL, USA). In all cases, $P \leq 0.05$ was considered statistically significant.

Results

The patients were randomly divided into the following two subgroups based on their drug consumption. The Consort statement flow diagram is presented in Figure 1.

Specific biochemistry tests

The baseline parameters were not significantly different between the two groups before treatment. The levels of serum Hcy, TAC, MDA, vitamin B12, and folic acid were obtained and compared before and after metformin and inofolic treatment (Tables 1, 2). The assay showed the level of all parameters before starting the treatment within the normal range in both groups. According to the findings, after metformin treatment for 3 months mean Hcy (normal range: 3.7-13.9 µmol/L) concentration increased in the serum (P=0.02). Also after inofolic treatment for 3 months mean Hcy concentration decreased in the serum but not significantly. The correlation between metformin and inofolic consumption with serum malondialdehyde concentration did not show a noticeable difference, but despite insignificant changes, it revealed a relative tendency to decrease serum malondialdehyde levels with inofolic consumption after the aforesaid time (P=0.12). Correlation between metformin consumption and total plasma antioxidant capacity showed a remarkable decrease in the TAC levels (485 Mm) in group A (P=0.01) but no substantial difference was observed in the serum TAC density after supplementation with inofolic in the group B. In addition, the levels of vitamin B12 did not show a significant difference in the subgroups after treatments. The mean serum level of folic acid was considerably increased in both groups (P=0.08) and indicated the same effect on the mean folic acid content.

Table 1: Basal clinical parameters of infertile PCOS patients in metformin and inofolic groups

Variables	Metformin	Inofolic	P value
Patients (n)	40	40	-
Age (Y)	35.37 ± 5.4[a]	34.21 ± 4.9[a]	0.07
BMI (kg/m^2)	27.82 ± 3.4[a]	28.43 ± 2.3[a]	0.1
Hcy (µmol/L)	12.21 ± 4.17[a]	11.43 ± 3.23[a]	0.06
Folic acid (ng/mL)	10.43 ± 5.86[a]	9.18 ± 4.41[a]	0.2
Vitamin B12 (Pg/mL)	219 ± 8.45[a]	220 ± 7.03[a]	0.09
TAC (µm)	531.34 ± 6.75[a]	584.82 ± 5.07[a]	0.1
MDA (nmol/mL)	4.86 ± 1.74[a]	4.11 ± 1.68[a]	0.1

[a]; In each column indicate no significant (P<0.05) difference. BMI; Body mass index, Hcy; Homocysteine, TAC; Total antioxidant capacity, and MDA; Malondialdehyde.

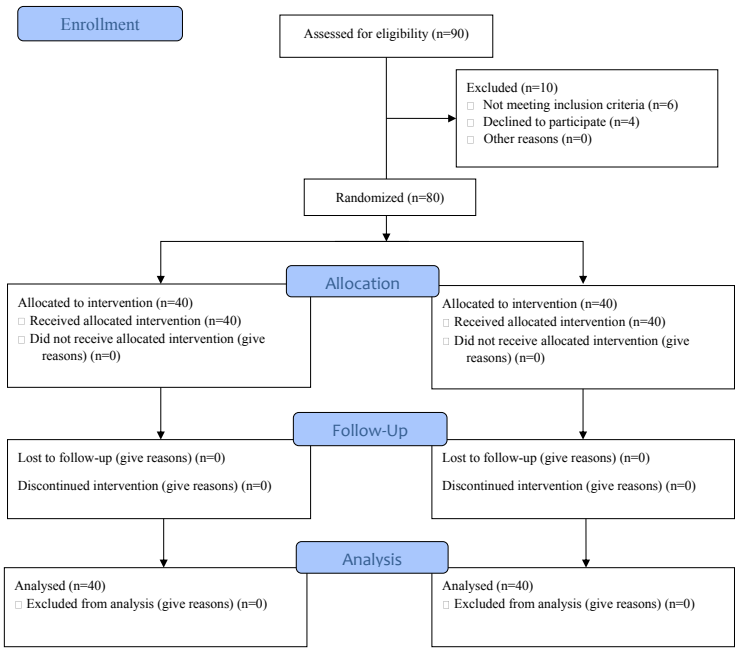

Fig.1: Consort flow diagram.

Table 2: Clinical parameters of infertile PCOS patients in metformin and inofolic groups before and after treatment

Variables	Group	Before treatment	After treatment	Difference	P value
Hcy (µmol/L)	A	12.21 ± 4.17a	13.39 ± 4.54[b]	1.18 ± 0.56	0.02
	B	11.43 ± 3.23[a]	10.94 ± 2.14[a]	-0.43 ± 0.14	
Folic acid (ng/ mL)	A	10.43 ± 5.86[a]	15.95 ± 6.31[b]	5.52 ± 1.13	0.04
	B	9.18 ± 4.41[a]	15.37 ± 5.7[b]	6.19 ± 1.25	
Vitamin B12 (Pg/mL)	A	219 ± 8.45[a]	237.4 ± 7.46[a]	18.40 ± 4.21	0.08
	B	220 ± 7.03[a]	227.29 ± 8.37[a]	7.29 ± 0.76	
TAC (µm)	A	531.34 ± 6.75[a]	485.87 ± 6.42[b]	-45.47 ± 6.16	0.01
	B	584.82 ± 5.07[a]	586.25 ± 7.8[a]	1.43 ± 0.37	
MDA (nmol/mL)	A	4.86 ± 1.74[a]	5.12 ± 1.5[a]	0.26 ± 0.08	0.12
	B	4.11 ± 1.68[a]	3.69 ± 1.31[a]	-0.42 ± 0.12	

Data are presented as mean ± SD. Assessed by paired t test. A; Metformin group, B; Inofolic group, Hcy; Homocysteine, TAC; Total antioxidant capacity, MDA; Malondialdehyde, and PCOS; Poly cystic ovary syndrome.

Discussion

Various studies have indicated that PCOS patients mostly experience high levels of androgen, insulin resistance, hyperinsulinemia, high level of estrogen and obesity that can affect metabolic parameters and oxidative stress (1). Previous studies have indicated that ROS and oxidative stress biomarkers show an increased amount of oxidative stress in PCOS patients (23). Previously, scientists declared a folic acid and vitamin B12 deficiency after a long-term metformin therapy in type 2 diabetes and PCOS patients. High levels of Hcy in PCOS patients might be explicable by these traits (6). Several studies have shown that serum folate depletion is one of the reasons for decreased fertility in women due to lack of ovulation (24). The mechanism by which folic acid affects fetal protection is not known, but may be due to the regulation of Hcy metabolism (9). Methylenetetrahydrofolate reductase, (MTHFR), is an enzyme that breaks down the amino acid homocysteine. The *MTHFR* gene that codes this enzyme has the potential to mutate, which can either interfere with the enzyme's ability to function normally or completely inactivate it. These mutations can lead to high levels of Hcy in the blood, which may contribute in numerous health conditions (7). Hcy is a cytotoxic amino acid that is catalyzed by multiple enzymes along with the cofactors such as folic acid. This study was designed with 80 infertile PCOS women to appraisal the effect of metformin and inofolic consumption on the alteration of serum Hcy, folic acid, vitamin B12, TAC, and MDA concentration.

Regidor et al. (25) study, reported there was 15% increase in pregnancy rates and significant decrease in testosterone and dramatic increase in progesterone levels after folic acid intake in PCOS women. Also, the patients who were taking myoinositol showed a higher rate of fertility and ovulation in this study. In a similar study, a 4-month treatment with metformin in patients with type 2 diabetes showed a dramatic decrease in serum folate concentration. The results of both studies are in contrast to our observations. There is no evidence of increase in serum folic acid concentrations during metformin therapy in previous studies, which is in contrast to our findings and could be due to the comparison of folic acid with metformin consumption.

On the other hand, our results in the present study indicated a significant increase in serum Hcylevels with metformin. In a study by Esmaeilzadeh et al. (5) investigating Hcy levels after 4 to 6 months of metformin intake, the results showed that metformin significantly increased serum Hcy levels in women with PCOS, suggesting that elevated serum Hcy levels may be one of the inherent side effects of metformin in this group. Many studies have reported metformin as a common drug in type 2 diabetes, with increased circulating Hcy concentration probably by decreasing plasma vitamin B12 and folic acid (26-28). In addition, metformin analysis showed that higher doses of metformin (more than 2000 mg/day) were associated with increased serum Hcy levels compared to lower doses (8). These results are also consistent with the present study and show that the effects of increased Hcy on metformin use is dose-dependent. Therefore, inofolic is a safe complement in PCOS women compared to metformin and folic acid. Folic acid uptake also appears to decrease with increasing Hcy concentration.

Studies have shown that folic acid and vitamin B12 levels are highly correlated, both of which affect reproductive quality and the health of women and fetuses. Folic acid and vitamin B12 are also absorbed in the body, but folic acid intake and serum concentrations are not mentioned in any of the related studies, and the results are consistent with our studies that metformin and inofolic consumption significantly increased folic acid levels. It did not show the amount of vitamin B12 in the serum (29). The results of our present study are probably because of long-term use of metformin on serum vitamin B12 concentration and suggest that the use of this drug on a temporary and short-term basis has no effect on vitamin B12 levels, thus it's administration can reduce related complications.

The present study showed a relative tendency to decrease serum malondialdehyde levels in inofolic compared to metformin consumption. Therefore, inofolice could reduce oxidative stress and its consequences such as

abnormalities in PCOS patients.

In a study conducted by Bułdak et al. (30) the effect of metformin on malondialdehyde in blood monocytes was examined on healthy non-smoker individuals and found that metformin reduced malondialdehyde in individuals. It is likely that the greater the sample size, the greater the decrease in malondialdehyde after ingestion. Since malondialdehyde increases in oxidative stress and decreases in inofolic group, therefore inofolic it can reduce the effects of oxidative stress in PCOS women and eventually can improve ovulation in these patients.

In one study, oxidative stress was measured in women with PCOS after taking myoinositol and metformin. The results showed no effect of metformin after 12 weeks of administration on malondialdehyde levels, which is consistent with our observations (31).

One study on diabetic rats found that 4 to 6 weeks of metformin prevented the increase in TAC in either treatment, monotherapy or combination therapy with insulin. This may be due to the action of metformin in controlling ROS production in diabetic rats, which is consistent with our observations (32). In another study in by Li et al. (6) that examined the levels of oxidative stress and Hcy in women with PCOS, the results showed a decrease in the TAC after metformin use in lean PCOS women. These observations are consistent with our results.

Conclusion

Considering the effect of metformin on serum Hcy levels and remarkable decrease in plasma TAC in infertile PCOS women, repeated monitoring of Hcy levels is recommended in patients with metformin intake and also suggest the use of inofolic and other medications containing inositol can be prescribed instead of metformin.

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Authors' Contributions

S.M.P., M.A.B., S.J.; Contributed to conception and design. S.M.P., N.A.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. M.A.B., S.J.; Were responsible for overall supervision. S.M.P., M.A.B; Drafted the manuscript, which was revised by A.K. and M.A.B. H.N.; Contributed to design and experimental work. A.K.; Contributed to data and statistical analysis and revise the manuscript. All authors read and approved the final manuscript.

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